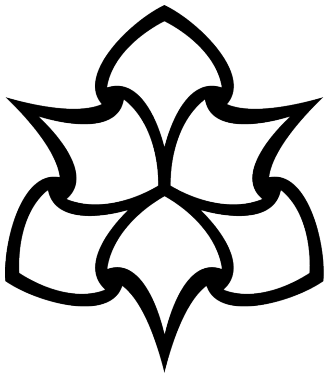

Revised submission. 'Differential effects of resveratrol on the dilator responses of femoral arteries, *ex vivo'*.

Running title: Differential effects of resveratrol on dilator function

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Abstract

Resveratrol is a plant-derived phytoalexin with antioxidant, anti-inflammatory and cardio-protective properties and may be a promising therapeutic intervention strategy in cardiovascular disease. Here, we investigated the acute direct effects of trans-resveratrol (RV), on acetylcholine (ACh)-induced and flow-mediated dilation (FMD) of isolated pressurized femoral arteries of young (4-month-old) and old (26-month-old) mice. Vessel exposure to RV enhanced ACh (0.01- 1.0 mM)-induced dilation ($p<0.05$), but not FMD (@ 5-10 $\mu L \cdot min^{-1}$) ($p<0.05$) in both young and old mice. After RV incubation, acute nitric oxide (NO) production by cultured endothelial cells was increased in response to 0.01 mM ACh, but reduced by flow (5-10 $\mu L \cdot min^{-1}$; $p<0.05$). In isolated femoral arteries from endothelial nitric oxide synthase knockout ($eNOS^{-/-}$) mice, RV had no overall effect on flow mediated dilation, but potentiated ACh induced dilation, that was completely abolished by potassium channel blockers, Apamin and Tram 34 ($p<0.01$). We demonstrate that the non-metabolised form of RV stimulates ACh-induced dilation via the NO and EDHF pathways, but not FMD by interaction with the cyclo-oxygenase pathway. Our findings have important implications in the use of RV (for both young and aged) under 'normal' non-diseased physiological states.

1. Introduction

Cardiovascular disease (CVD) is associated with compromised endothelial function, characterised by decreased capacity of the blood vessels to dilate in response to vasodilator stimuli. During ageing a reduced endothelium-dependent dilation in response to acetylcholine (ACh) and intraluminal flow has been reported in the aorta and mesenteric arteries in rodents, which has been linked with a reduced bioavailability of nitric oxide (NO) [1; 2; 3]. This reduced NO availability can be attributed to a reduced nitric oxide synthase (eNOS) activity [4; 5; 6] and elevated levels of reactive oxygen species (ROS) [7; 8; 9]. The regulation of the diameter of small arteries is crucial for the regulation of blood pressure and perfusion of tissues. Improving dilatory capacity hence improves oxygen and nutrient delivery to the muscle, supporting muscle function, growth and adaptation to mechanical and nutritional stimuli [10]. Consequently, improving endothelial function of the arteries feeding the skeletal muscle may well prove to be a target to combat age-related degenerative conditions such as sarcopenia [11; 12].

Resveratrol (RV) is a polyphenol with anti-inflammatory and anti-oxidant properties involving AMP-activated protein kinase (AMPK) and silent mating type information regulation 2 homolog (SIRT) 1 activation [13], and it has been reported to reduce markers of inflammation and levels of oxidised low-density lipoproteins (LDLs) in large clinical studies [14; 15]. RV (1-10 μM) can promote vasorelaxation and improve endothelium-dependent ACh-induced dilation of pre-constricted rat aortic rings *ex-vivo* [16]. In addition, RV restored endothelium-dependent ACh-induced dilation in isolated mouse aorta impaired

by high glucose [17]. These responses are accompanied by reductions in blood pressure in spontaneously hypertensive rats [18]. Both vasodilation and blood pressure reduction are thought to be primarily, though not completely, mediated by increases in the expression and activity of eNOS, and the bioavailability of NO, though this is also dependent on the vessel size and particular vascular bed [18; 19; 20; 21].

In elderly patients with coronary artery disease [22] and metabolic syndrome [23], chronic supplementation with RV (10 or 100 $mg \cdot day^{-1}$ for three months, respectively) has been shown to improve flow-mediated dilation (FMD) of the brachial artery. Other studies, however, question the efficacy of RV in improving cardiovascular parameters, particularly as it has been shown to blunt the benefits triggered by physical exercise [24]. The discrepancy in findings might be attributed to the various doses of RV used (pharmacological vs dietary); the different types of vascular beds, the different stimuli (pharmacological vs mechanical), and the type of exercise (aerobic vs. resistance) investigated [25; 26].. In humans, peak blood plasma concentrations of RV are usually below 10 $ng \cdot mL^{-1}$ (40 nM) after a dietary dose [15], and up to 500 $ng \cdot mL^{-1}$ (2.1 μM) after RV supplementation [27]. Despite high levels of RV absorption from the gut (>70%) [15], low plasma concentrations are likely due to extensive RV gut metabolism by intestinal and hepatic enzymes [28; 29]. Resulting RV metabolites can reach peak plasma concentrations of 400-1700 $ng \cdot mL^{-1}$ [30]. Hence, whether RV or its metabolites can influence dilator function, particularly for small size arteries, which play a pivotal role in diameter regulation, remains unclear. In addition, it was demonstrated *in-vitro* that RV, but not any of the metabolites, increased the activity and gene

expression of eNOS in endothelial cells (EC) and reduces intracellular ROS levels [31]. Consequently, enhanced delivery of unmetabolised RV into blood plasma, tissues and to molecular targets is currently being explored via numerous pathways such as intravenous delivery of RV by encapsulation in liposomes [32], via solid lipid nanoparticles [33], and/or by increasing the oral bioavailability with RV-containing micro-emulsions, or nano-particulate colloidal systems [34; 35]. Slow release formulations are also being developed for both oral and intravenous delivery to overcome the short plasma half-life and rapid urinary excretion of RV [33].

Due to the complicated interacting mechanisms that regulate arterial function *in vivo*, it is difficult to dissect out pathways and elucidate the direct effects of RV. Furthermore, as RV is metabolised in the gut, it is impossible to ascertain if the effects are attributed to RV itself or any of its metabolites. Here, using an *ex vivo* model, we have examined the acute effects of RV on the maximal dilatory response of the isolated pressurized mouse femoral artery from young and old mice, and from eNOS knock-out mice, to increasing doses of ACh and intraluminal flow. Stability of RV during pressure myography experiments was confirmed by HPLC analysis. Finally, to assess whether RV effects were attributable to enhanced NO release, we measured the production of NO in response to ACh and flow in cultured ECs. Our findings will have important implications in the use of RV as a nutraceutical strategy for improved dilator function.

2. Materials & Methods

2.1 *HPLC analysis of RV purity and stability*

RV was supplied in hard-shelled capsules (containing 330 mg RV, at a stated purity of >98%) by 21st Century Alternatives (UK). To verify the purity of RV, 2 x 1 mg of the powdered material in three individual capsules was extracted with aqueous ethanol (50% v/v), containing ascorbic acid (0.1% w/v), and taxifolin as internal standard (100 $mg \cdot mL^{-1}$). The mixture was vortexed for 0.5 min, placed in an ultrasonic water bath for 20 min, and vortexed again. After centrifugation at 13,000 rpm for 10 min (Microcentaur, MSE, London, UK) supernatant was collected, the extraction was repeated, and supernatants were combined. RV content was determined in diluted extract (1 in 10) using a Shimadzu HPLC system with a Prominence delivery system (LC-20AB binary pump, SIL-20ACHT autosampler set to 8°C and CTO-20A column oven set to 35°C) connected to a NexeraX2 (SPD-M30A) diode array detector, scanning between 190-700 nm. RV was separated on a C18 column (150 mm x 2.1 mm, 2.6 µm, ThermoFisher Scientific, Altringham, UK) fitted with a low volume "Krud catcher" (Phenomenex UK) on a binary gradient of aqueous HPLC grade acetonitrile (5 *vs*. 95% v/v, solvents A and B, respectively) plus 0.2% formic acid (v/v) running at a flow rate of 0.3 $mL \cdot min^{-1}$, using a gradient of 0 – 5 % solvent B from 0 to 5 min, 5 – 20% from 5 to 20 min, 20 – 95% from 20 to 25 min, 95% from 29 to 33 min, 95 – 0% from 33 to 34 min, 0% from 34 to 37 min. Taxifolin and RV were identified at 289 nm and 307 nm, respectively, and. quantified via an 8-point standard curve ranging from 3.75–150 $mg \cdot mL^{-1}$ [36]. All solvents were of HPLC grade or higher (Fisher Scientific, Loughborough, UK). Sodium azide, EDTA, ascorbic acid and *trans* RV reference standard were obtained from Sigma (Dorset, UK).

Taxifolin was obtained from Extrasynthese (Product code 1359S; Vichy, France). All reference standards were of HPLC grade (>95%).

To assess stability of RV during experiments, prior to, and following incubation of the femoral segments, aliquots of the RV containing pressure myograph incubation medium was collected. RV was stabilised by addition of a buffer (10% v/v) containing NaH_2PO_4 (0.4 M at pH 3.6), ascorbic acid (20% w/v) and EDTA (0.1% w/v), and samples were stored at -20°C. samples were diluted 1 in 10 (v/v), taxifolin was added as internal standard (1 $mg \cdot mL^{-1}$) and HPLC analysis was conducted as described above.

2.2 Animal tissue

Pathogen-free, 4- (young) and 26-month-old (old) male C57BL/6 mice were used. The animals were obtained from Charles Rivers Laboratories (UK) where they were maintained until one week before the experiments. The body mass was 28 ± 1 g in young (age 16 weeks n= 6-11) and 30 ± 1 g in old (age 104 weeks, n=6-8) animals. They were humanely euthanized by cervical dislocation in accordance with the 'Animals (Scientific Procedures) Act 1986', Institutional guidelines (ethics reference number SE131413) and in accordance with EU directive guidelines. Mice lacking the endothelial isoform of nitric oxide synthase ($eNOS^{-/-}$ mice) were obtained from Jackson Laboratories and maintained at the University of Manchester under project license (PPL) 40/8504.

2.3 Pressure myography

Segments of the femoral artery (proximal location near the groin in the area close to the inguinal ligament; ~3 mm long) were finely dissected (the fat tissue layer and connective tissue surrounding each vessel were removed) and mounted between two glass cannulae on a pressure myograph chamber (Living Systems Instrumentation, Burlington, VT, USA). The preparation was checked for leaks and readjusted if required. Arteries were initially pressurised to an intravascular (luminal) pressure of 60 mmHg [37]and maintained at that pressure using a pressure servo-control unit (Living Systems, Burlington, USA). The arteries were equilibrated and constantly superfused in a continuous source of physiological salt solution (PSS) [composition [mM]: 119 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.1 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 \cdot 2H_2O$] at 37°C, pH 7.4, gassed with 95% air-5% CO_2. The chamber was placed over an inverted microscope (Nikon Eclipse TS100, Japan) to constantly measure the internal diameter of the vessel using a video dimension analyzer, with data recorded on a computer using Chartlab 5 software (Powerlab system, AD Instruments, UK). The viability of the femoral arteries was assessed for their ability to constrict in response to a high potassium physiological salt solution (KPSS; composition [mM]: 119 NaCl, 60 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.17 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 \cdot 2H_2O$; pH 7.4) and to the alpha-adrenergic receptor agonist phenylephrine (Phenyephrine hydrochloride (Phe) 10 μM; 1002640296, Sigma Aldrich, UK). Segments of the femoral artery were incubated with RV 45 μM (0.1% dimethyl sulfoxide (DMSO)) or PSS (controls; 0.1% DMSO) for one hour. The dose of RV was chosen based on previous *in-vitro* studies showing positive effects of RV on a broad range of vascular and metabolic parameters [38; 39; 40].

To investigate the contribution of known vasodilators to ACh-induced dilation and FMD we incubated segments of the artery with inhibitors of vasodilator pathways: L-NG-nitro-L-arginine (L-NNA, an inhibitor of nitric oxide synthase; 100 μM; 101043560, Sigma Aldrich, UK); indomethacin (a nonselective inhibitor of cyclooxygenases; 10 μM; I7378, Sigma Aldrich, UK); or the combination of apamin (a selective blocker of small-conductance calcium-activated potassium channels; 100 nM; A1289, Sigma Aldrich, UK) and Tram 34 (a selective blocker of intermediate-conductance calcium-activated potassium channels; 1 μM; T6700, Sigma Aldrich, UK) alone or in combination with RV (45 μM). For control vessels, they were superfused in PSS as above. After a 1-hour incubation in RV or PSS, dilator responses in pre-constricted vessels (Phe, 10 μM) in the presence of inhibitors, to ACh (10^{-9}-10^{-3} M; Acetylchlorine hydrochloride, 101873875, Sigma Aldrich, UK) and intraluminal flow (5-10 $\mu L \cdot min^{-1}$) were determined. Intraluminal flow was introduced for 4-5 minutes at each flow rate with a peristaltic pump (flow control pump FC, Living Systems Instrumentation, Burlington, VT, USA) connected to the right cannula (distal end of the vessel) of the chamber. The pressure gradient within the vessel was constantly monitored with a PM/4 perfusion pressure monitor (Living Systems Instrumentation, Burlington, VT, USA). At the end of each experiment, endothelial-independent responses were assessed using the nitric oxide donor, sodium nitroprusside (SNP, 101434502, Sigma Aldrich). Arteries were then superfused with calcium (Ca^{2+})-free PSS containing 2 mM EGTA for 20 minutes to obtain passive diameters. Unless otherwise stated, each group of experiments consisted of at least five animals (1 vessel per animal). All drugs and reagents were obtained from Sigma-Aldrich (Poole, UK), unless otherwise stated. The experimental groups were divided as: young control (YC), young RV (YRV), old control (OC), and old RV (ORV).

2.4 Cell culture

To examine the influence of RV on endothelial cell viability and production of NO, human coronary artery endothelial cells (HCAEC, Promo cell, C-12221) were grown as a monolayer at 37°C under 5% CO_2 atmosphere in EC growth medium MV (Promo cell, C-22020) supplemented with 5% foetal calf serum, 4 $\mu L \cdot mL^{-1}$ growth medium, 0.5 ng/mL^{-1} vascular endothelial growth factor, 10 $ng \cdot mL^{-1}$ epidermal growth factor and 90 $\mu g \cdot mL^{-1}$ heparin. Before use, cells were washed three times in phosphate buffered saline (PBS) and then incubated with 0.1% trypsin for three minutes to detach them from the flask surface, centrifuged for 3 min at 220 *g*, and re-suspended in growth medium for seeding. Cells were seeded onto Nunc Thermanox coverslips (10,000 cells/coverslip; 13 mm diameter) (Thermo Fisher Scientific, USA) and allowed to proliferate for 24 hours (approximately 90% confluency). Half of the coverslips were kept in the well plate to perform the ACh assay while the other overslips were transferred into a millifluidic system made up of a bioreactor chamber (Kirkstall, UK) attached with a flow pump system in which increased flow was introduced [41] as indicated in the section below. All reagents were obtained from Sigma-Aldrich (Poole, UK), unless otherwise stated.

2.5 Determination of NO production

Cells were exposed to RV 45 μM dissolved in 0.1% DMSO and growth medium for one hour to simulate the incubation period in the pressure myograph. Control cultures were treated with growth medium (0.1% DMSO) alone. After the incubation, the supernatant was collected and the cultured cells exposed to ACh 10^{-5} M for 2-10 minutes, or flow rates of 5-

10 μL·min^{-1}, calculated at 5.59 x 10^{-4} dyn/cm^2 (representing physiological rates of interstitial flow) [41], for 3-9 minutes. Following exposure to ACh or increased flow, the supernatant was collected and the production of NO quantified using the Griess assay (23479, Sigma Aldrich, UK). Briefly, samples were immediately incubated with flavin adenine dinucleotide (50 μM), reduced β-nicotinamide adenine dinucleotide phosphate (500 μM) and nitrate reductase from *aspergillus* species (1 u·mL^{-1}) for 15 minutes at 37°C. Then, the samples were incubated with lactate dehydrogenase (100 u·mL^{-1}) and sodium pyruvate (100 mM) for a further five minutes at 37°C.. Equal volumes of sample and Griess reagent (1% sulfanilamide in 2.5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) were incubated at room temperature for 10 minutes. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA). The content of nitrite was calculated based on a standard curve constructed with $NaNO_2$ at the concentrations ranging from 3.12 to 400 μM. The assay sensitivity is approximately 125 pMoL [42]. All reagents used in the cell culture experiments were prepared in the growth medium mentioned above and acquired from Sigma (Dorset, UK), unless otherwise stated.

We determined cell viability with the Alamar Blue assay (DAL 1025, Thermofisher Scientific, UK), which quantifies metabolically active cells. Briefly, the samples were washed for five minutes in PBS and incubated in a solution of 2 μM calcein-AM (live) and 4 μM ethidium homodimer-1 (dead) solution in PBS for 30 minutes. After rinsing, the samples were imaged with an inverted fluorescence microscope (Leica DMI6000B; Leica Microsystems, Germany)

using a conventional fluorescein long pass filter. The quantification of the cells was done with Image J software (US National Institutes of Health).

2.6 Statistical Analysis

All statistical analysis was performed using SPSS software version 20 (IBM, Chicago, Illinois, USA). The dilator responses elicited by ACh and intraluminal flow in the presence of RV or PSS are expressed as percentage dilation from the pre-constricted value. ACh concentration response curves and flow responses from the segments of the femoral artery in the pressure myograph and the cultured cells were assessed, independently, using a two-way repeated-measures analysis of variance (ANOVA) with as within factors: flow (three levels: 5, 8 and 10 $\mu L \cdot min^{-1}$) or ACh concentration, and as between factors: RV, age, and/or treatment. If main effects or interactions were found, subsequent unpaired t-tests were performed to locate the differences. Values of $p<0.05$ were considered significant. Data are represented as means ± standard error of mean (SEM) unless stated otherwise.

3 Results

3.1 RV purity and stability

Purity of RV used in this study was 97 ± 13 % (± SD) (Figure S1). The concentration of RV at the beginning of the pressure myography experiments was (mean ± SD) 47.0 ± 1.6 μM and dropped to 34.4 ± 1.5 μM following the 1-hour circulation in the myograph chamber,

indicating a 27.3 ± 4.6% (n=3) loss of RV between pre- and post-incubation. No RV metabolites (stilbenoid metabolites) were detected via HPLC analysis, indicating that the only bioactive compound present was the RV aglycone.

3.2 Constrictor responses to KPSS and Phe

The initial diameter of the arteries used in these studies did not differ significantly between groups: 156 ± 20 μm (YC; n=12); 163 ± 9 μm (YRV; n=10); 150 ± 34 μm (OC; n=6), and 172 ± 38 μm (ORV; n=6) (Table S1 supplementary material). The degree of constriction caused by superfusion with 60 mM KPSS or 10 μM Phe was similar in isolated femoral arteries from young and old mice and was not significantly affected by incubation with RV (Figure S2).

3.3 Effects of RV on dilator responses

The ACh (10^{-9}-10^{-3} M)-induced concentration-dependent dilation in isolated pressurized femoral arteries was similar in young and old mice. Maximal responses were observed after 2-3 minutes. RV increased dilation to ACh in both age groups when compared with their respective controls ($p<0.05$) (Figure 1A)(Table S1, supplementary material). Smooth muscle cell sensitivity to NO was determined in young mice, by assessing the endothelium-independent responses to SNP and found to be unaffected by the absence/presence of RV (101.32 ± 5.30 [n=6] and 94.36 ± 9.42 [n=4] in the absence/ presence of RV, respectively).

3.4 ***Characterization of the ACh-induced dilation***

To ascertain the mechanisms by which RV can improve dilator responses of the femoral artery, we firstly characterised the ACh-induced dilator components in vessels from young and old mice, without pre-incubation with RV, using dilator pathway inhibitors. L-NNA reduced dilation (Fig. 1A; $p<0.01$). The absence of a significant age and L-NNA interaction indicates that this effect was similar in vessels from young and old mice (Figure 1B, C). As the dilator components were similar in vessels from young and old mice, we investigated the effect of RV in vessels from young mice only. In both control and RV co-incubated vessels, L-NNA (Figure 1D) and apamin + Tram 34 (Figure 1E) led to a reduction in dilator responses ($p<0.02$). Similar to control, indomethacin did not significantly inhibit ACh-induced dilation in vessels co-incubated in RV (Figure 1F).

3.5 Characterization of FMD

Introduction of intraluminal flow resulted in dilation of arteries from both young and old mice. This was significantly greater at 10 $\mu L \cdot min^{-1}$ in comparison to 5 $\mu L \cdot min^{-1}$ for vessels from both the young and aged mice ($p<0.05$). Maximal responses were observed after approximately 1-2 minutes (Figure 2A; An increase in flow rate (to 100 $\mu L \cdot min^{-1}$) did not lead to any measurable increase in diameter for any of the vessels tested). Similar to ACh-induced dilation, FMD did not differ significantly between vessels from young and old mice (Figure 2B). There was a significant interaction ($p<0.01$) between the interventions and age. This was reflected by the fact that only in vessels from old mice, Apamin + Tram 34 inhibited

FMD (at 8 μL·min^{-1}) ($p<0.05$), while L-NNA and Indomethacin led to significant reduction in flow at 8 and 10 μL·min^{-1} ($p<0.01$)(Fig 2C & D).

3.6 Effects of RV on FMD

There was a main effect of RV on FMD ($P<0.01$), but the interaction between flow and the presence or absence of RV ($p<0.01$) was reflected by lack of flow-induced dilation at the higher flow rates, but not entirely at the lower flow rate in vessels from both young and old mice (Figure 2). This observation may explain why addition of L-NNA, apamin + Tram 34 or indomethacin to vessels co-incubated with RV did not induce further reductions in FMD (Fig. S3; $p<0.05$).

3.7 Effects of RV on dilation in arteries from eNOS knock-out mice

To further understand which dilator pathways are stimulated or inhibited by RV, we examined responses of femoral arteries from eNOS knock-out mice. All vessels constricted to 60 mM KPSS and 10 μM phenylephrine to a similar degree as in vessels from wildtype mice (RV, PSS, RV plus inhibitors). Vessels dilated minimally in response to ACh (10^{-9} – 10^{-3} M), and RV incubation led to an improved dilation to a level similar to that in vessels from wildtype mice incubated with ACh (Fig. 3) dilation of arteries taken from C57 control mice after PSS incubation. We observed a significant main effect of treatment ($p=0.002$) and animal model ($p<0.001$), with interaction between them (Fig 3). Subsequent independent t-test analyses demonstrate that co-incubation in RV and potassium channel inhibitors

(apamin and Tram 34), led to a significant reduction in dilator responses (at ACh 10^{-7} -10^{-5} M, $p<0.05$; and at ACh 10^{-4} -10^{-3} M, $p<0.01$) (Fig. 3). Introduction of intraluminal flow resulted in dilation of arteries from the eNOS KO mice. Incubation with RV or RV plus apamin and Tram 34 inhibitors, had no overall effect on dilator responses. There was no overall main effect of either treatment or animal model.

3.8 Effects of RV on NO production

To assess the direct influence of acute RV exposure on ECs and its effect on NO production, isolated ECs were stimulated with ACh and flow over the same time that mimics the temporal responses observed with pressure myography. We observed that the production of NO in response to ACh is highest during the first two minutes with a steady decrease over time. Incubation with RV increased the production of NO at two minutes ($p<0.05$; Fig 4). During flow conditions, the production of NO is transiently elevated ($p<0.05$), but less so during incubation with RV ($p<0.05$; Fig. 4). The reduction in the production of NO by EC during flow conditions, in the presence of RV, was not attributable to reduced cell viability, which was similar amongst all groups (mean ± SD) 96 ± 2% (Fig. 4).

4 Discussion

While we demonstrate that dilator responses of isolated mouse femoral arteries to ACh and intraluminal flow are not compromised with age, our key finding is that acute *ex-vivo* exposure to RV enhances ACh-induced dilation, but not FMD in arteries from both young and

old mice. The differential effects on ACh-induced dilation and FMD suggest that RV might impair flow-mediated endothelial mechano-transduction at low shear stress but potentiates the release of ACh-induced endothelial derived mediators, by stimulating the endothelial-derived hyperpolarizing factor (EDHF) and NO pathways. Using isolated endothelial cells in culture, we show ACh-induced release of NO. These effects can be ascribed to RV itself as *trans*-RV appeared stable during pressure myography experiments and we found no conversion to *cis*-RV, or any other metabolite.

4.1 Endothelial function and ageing

We observed no attenuation in dilation (a marker of endothelial function) to either ACh or intraluminal flow in the femoral artery of aged mice (Figures 1A and 2B). While some studies indicate that ageing blunts NO-dependent relaxation, hence compromising endothelium-mediated dilation of the aorta [43], mesenteric artery [44] and coronary arterioles of rats [45], our findings are in agreement with those reported by Sinkler and Segal [6], demonstrating that the maximal internal diameter induced by ACh of the feeding artery of the gluteus maximus muscle from 26-month old mice did not differ from their 4-month old counterparts [6]. This was accompanied by similar constrictor responses to Phe [4]. In fact, second- and third-order arterioles from the old mice showed enhanced dilation to doses of ACh 10^{-7} M and higher. Furthermore, ACh-induced dilation was proportional to that evoked by sodium nitroprusside. Together, this suggests that the endothelium was fully able to drive vascular smooth muscle relaxation without any age-related complications [6]. These findings are in agreement with those reported by Bearden and colleagues [4], who demonstrated a

conserved architecture of arteriolar networks in the gluteus maximus muscle of young (3 months), adult (12 months) and old (20 months) mice. This was accompanied by similar dilator and constrictor responses to ACh and Phe, respectively [4]. More recently, Nguyen and colleagues [46] showed that the ACh-induced dilation of the isolated and pressurized femoral artery from 9 and 12-mo C57BL/6 mice was proportional amongst sedentary, catechin-treated (an antioxidant with putative cardiovascular properties) and exercising mice [46]. Only in the presence of LNNA was ACh-induced dilation lower in the 12-mo sedentary mice which supports the notion of an age- and vascular bed-dependent contribution of specific vasodilators in response to ACh [46]. In addition to femoral artery responses, we also demonstrate that the aortic vessel responses to ACh-stimulation are similar in our young and aged mice (see supplementary material, Fig. S4)

The ACh-induced and flow-mediated endothelium-dependent dilation of arteries depend on the interplay of NO, EDHFs and prostacyclin (PGI_2) [47]. Therefore, we characterized the dilator response of the mouse femoral artery to ACh and intraluminal flow by inhibiting the production of such molecules. The ACh-induced dilation was reduced upon treatment with L-NNA or the combination of apamin + Tram 34 in both young and old mice while indomethacin had little impact on maximal diameter, indicating that NO significantly contributes to the ACh-induced dilation with no, or an insignificant, contribution of PGI_2 (Figures 1B & C).

In addition to ACh-induced dilation, we also assessed endothelial function by means of FMD. The dilation of arteries in response to increases in wall shear stress is more physiologically relevant than agonist stimulation [37], and is considered a clinical measure of endothelial-dependent dilation [48]. Similar to ACh-induced dilation, we found preserved FMD with age and observed significant differences in the contribution of endothelial factors to maximal FMD with ageing. Of interest is the fact that FMD seems to rely on NO and EDHFs in the young with little contribution of PGI_2. This is in contrast with the aged arteries in which PGI_2 is an important contributor to FMD (Figures 2B & C). This may be an early hallmark of vascular dysfunction in old age. Our findings are partially in agreement with a previous report that studied age-related changes in FMD of the mouse femoral artery [49], but not in soleus feed arteries from the rat [50]. In line with our observation, using high-frequency ultrasound, Schuler and colleagues demonstrated that FMD of the mouse femoral artery is mostly NO-dependent and that eicosanoids play little, if any, role in dilation in 12-week-old male mice while the NO-dependent component of FMD decreased with age [49]. It has been proposed that such changes reflect back-up mechanisms that compensate for the loss of NO bioavailability, or release of other endothelial factors, particularly during the late stages of life or in pathological states [51; 52; 53]. Our findings thus confirm previous studies that indicate an age-related shift from NO to a larger dependence on PGI_2 in vessel dilation of isolated pressurized arteries in response to intraluminal flow. Reduced dilator responses in vessels from aged mice have also been attributed to increased wall stiffness and/ or reduced compliance in mesenteric/femoral arteries from 23m old mice [5; 54]. In our hands, ACh-dependent dilation was similar in aortic vessels from both young and old mice and many be a consequence of activation of compensatory dilator pathways.

4.2 Effects of RV on vessel dilation

We found that regardless of age, RV markedly improved ACh-induced dilation. It has been shown *ex-vivo* that RV induced vasodilation in noradrenaline- and potassium chloride- pre-constricted mesenteric and uterine arteries from female guinea-pigs (5-50 μM) [55] and mesenteric arteries of male F344 x Brown Norway rats (0.01-100 μM) [1] and obese Wistar rats (5-35 μM) [56]. In the latter study, ACh-induced dilation was blunted in the arteries from obese rats, which led the authors to conclude that RV might also promote dilation by means of endothelium-independent mechanisms [56]. In our study, RV potentiated ACh-induced dilation of the isolated pressurized femoral artery from young and aged healthy mice (Fig. 1A). Such effects were abolished upon co-incubation of the femoral segments with RV and L-NNA or apamin + Tram 34, which indicates that RV-improved dilation is mediated by NO and EDHFs (Figures 1D-F). Our isolated endothelial cell culture studies demonstrate that RV pre-incubation increased ACh-induced NO release, further confirming RV's potentiation of ACh-induced NO production. In isolated femoral arteries from the eNOS KO mice, where there is no NO production due to the absence of the eNOS enzyme, RV potentiated the ACh-induced dilator responses (Fig. 3). When RV was co-incubated in Apamin and Tram 34, which block the EDHF pathway, dilation was completely abolished. Previous evidence has suggested that in the absence of the eNOS enzyme, EDHF responses become most prominent as a compensatory response [57; 58]. Here, we demonstrate that RV potentiates dilation by activating small (SK_{Ca}) and intermediate conductance (IK_{Ca}) potassium channels leading to activation of the EDHF pathway in isolated small femoral arteries. The mechanism of RV induced EDHF-mediated relaxation has been shown to involve redox-sensitive activation of the phosphatidylinositol (PI) 3-kinase/Akt signal transduction pathway [59]. In femoral

arteries from wildtype mice, we used the non-selective cyclooxygenase (COX) inhibitor, indomethacin, to block the release of PGI_2. In most blood vessels, the major source of PGI_2 is endothelial COX-1, while its release is differentially regulated by COX-2 [60]. Activation of COX-2 also induces the release of vasoconstrictor prostanoids and COX-2 inhibition in hypertension patients led to improved FMD [61]. Although RV has been shown to influence both COX-1 and 2 isoenzymes, it can directly bind to COX-2, inhibiting its activity [62]. The improved dilator responses observed after co-incubation of vessels with RV and indomethacin, at low ACh concentrations (Figure 1F), suggests that RV acts as a modulator of COX-mediated dilator function, influencing both dilator and constrictor pathways.

While RV markedly improved ACh-induced dilation, it compromised FMD. The mechanisms mediating compromised FMD by RV are likely to involve the inhibition of endothelial derived dilator factors since incubation of vessels, in the absence of RV, with L-NNA, apamin + Tram 34 or indomethacin resulted in significantly lower levels of dilation to intraluminal flow when compared to controls. We cannot exclude, however, the possibility that RV inhibits FMD by endothelium-independent mechanisms or by induction of endothelial derived vasoconstrictor factors (such as PGE_2) via COX-2 pathway. We are not aware of any other *ex-vivo* study that investigated the direct effects of RV on FMD in the isolated pressurized mouse femoral artery. Although RV has not been shown to reduce blood pressure (BP) regardless of dose or duration of treatment [63], it seems to have positive effects on FMD in some instances. For instance, RV (3 $mg \cdot kg^{-1} \cdot day^{-1}$ for six weeks) improved FMD of the femoral artery in rabbits fed a high-cholesterol diet [64]. Further, in middle-aged and older obese

humans with elevated BP, but not in normotensive subjects, acute (30-270 mg) and chronic (75 mg·day^{-1} for six weeks) supplementation with RV improved FMD of the brachial artery [65; 66]. We have recently demonstrated that RV can potentiate FMD where it is compromised in patients after coronary artery bypass grafting (CABG) surgery but blunted the response where the endothelial responses are intact in patients that had undergone percutaneous coronary intervention (PCI) intervention [67]. The exact constituent responsible for such effects *in vivo* was not identified in these studies, which is especially relevant since RV is metabolised in the gut when administered orally. This may explain the discrepancy with our observations, whereby our vascular effects are most likely mediated by non-metabolised *trans*-RV as we have observed no apparent stilbenoid metabolite peaks in our incubation medium after 1 hour of incubation with RV. The loss of RV is most likely due to oxidative breakdown or binding to the walls of the tubing and incubation bath.

Using our methodology, the flow rates established to achieve FMD, represented low shear stress, equating to 1-2 dyn/cm^2 within the isolated vessel (Fig. 4) [47]. Consequently, RV's influence on FMD may vary depending on the level of expression of mechanoreceptors, as well as the concentrations used. RV can bind to integrin and growth factor receptors directly influencing mechanotransduction pathways. For example, integrin αvβ3 contains a receptor site for RV, the binding of which has been shown to influence cellular function and induce apoptosis [68]. These effects were evident at RV concentration of 10 μM. The dose dependent effect of RV on EC uptake [69], proliferation and apoptosis is well documented [70; 71]. Using isolated endothelial cells in culture, Lee and colleagues demonstrated that while at low doses

RV (10 μM) promoted eNOS phosphorylation, thus increasing NO release, at high doses (100 μM), it led to down regulation of NO production, by acting via the FERM domain of the focal adhesion kinase (FAK) [72]. In terms of acute effects, the small degree of flow mediated constriction observed in our study in vessels from aged mice after RV incubation (at higher flow rates) may be due to activation of pro-contractile voltage gated calcium channels leading to smooth muscle cell depolarisation and consequent vessel constriction [73].

5 Conclusions

Our findings indicate that in the isolated pressurized mouse femoral artery the mechanisms that govern dilation differ according to type of stimulus (agonist *vs*. flow). The age-related shift in the contribution to flow-mediated dilation towards a larger role of PGI_2 in the old vessels is associated with unaltered levels of maximal dilation. Further, we show that *trans*-RV significantly enhanced ACh-induced dilation, but not FMD at shear stress equivalent to 1-2 dyn/cm^2. L-NNA significantly reduced ACh-induced dilation, suggesting that RV stimulates the NO pathway, which we further confirmed by demonstrating the release of NO by cultured endothelial cells after RV incubation. In the absence of the eNOS enzyme (in vessels from eNOS KO mice), RV's potentiation of dilation was completely abolished in the presence of inhibitors that block the small and intermediate conductance potassium channels, thus also implicating EDHF. The evaluation of vascular responses *ex-vivo* with pressure myography provides a well-controlled model for the understanding of the pathophysiological properties of blood vessels. Considering the clinical value of FMD, it is surprising that there is currently no generally accepted procedure in rodents that is in line with that in humans. Evidence attributing positive effects of RV to FMD in both humans and other animals is fairly recent

and not enough data have been gathered in this area. Our results show that in femoral arteries from 'normal' mice, RV does not potentiate FMD at the low flow rates tested. However, this may become relevant at high flow rates. Our findings have important implications in the direct delivery of RV as a therapeutic strategy to modulate dilator function, in diseased but not 'normal' states.

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Author Disclosure Statement

No competing financial interests exist.

List of abbreviations: acetylcholine (ACh); AMP-activated protein kinase (AMPK); blood pressure (BP); calcium (Ca^{2+}); cardiovascular disease (CVD); coronary artery bypass grafting (CABG); cyclooxygenase (COX) dimethyl sulfoxide (DMSO); endothelial cells (EC); endothelium derived hyperpolarizing factors (EDHFs); flow-mediated dilation (FMD); focal adhesion kinase (FAK); high-performance liquid chromatography (HPLC); high potassium physiological salt solution (KPSS); human coronary artery endothelial cells (HCAEC); L-NG-nitro-L-arginine (L-NNA); low-density lipoproteins (LDLs); nitric oxide (NO); endothelial nitric oxide synthase (eNOS); percutaneous coronary intervention (PCI); phenylephrine (Phe); physiological salt solution (PSS); phosphatidylinositol (PI); prostacyclin (PGI2); reactive oxygen species (ROS); resveratrol (RV); silent mating type information regulation 2 homolog (Sirtuin); standard error of mean (SEM); two-way repeated-measures analysis of variance (ANOVA).

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Figure legends

Figure 1. Acetylcholine (ACh) induced dilator responses in femoral arteries from wildtype young and old mice. ***A) Effects of resveratrol (RV) on ACh-induced dilation.*** *RV significantly improved dilation of the isolated pressurized femoral artery segments (*p<0.05). YC: young control (n = 6); YRV: young RV (n=6); OC: old control (n = 6); and ORV: old RV (n = 6).* ***B, C) Characterization of the dilator response to ACh.*** *Incubation of isolated pressurized femoral arteries from wildtype young (**B**) and old mice (**C**) with L-NG-nitro-L-arginine (L-NNA; 100 μM; n = 6 per group), indomethacin (10 μM; n = 6 per group), or the combination of apamin (100 nM) + Tram 34 (1 μM) (n = 6 and 8 for the young and old groups, respectively; *p<0.05).* ***D, E, F) Characterization of the dilator response to ACh in young mice in the presence of resveratrol (RV);*** *with (**D**) L-NG-nitro-L-arginine (L-NNA; 100 μM; n = 5); (**E**) apamin (100 nM) + Tram 34 (1 μM) (n = 5); or (**F**) Indomethacin (10 μM; n=5). Both L-NNA and apamin+Tram, but not Indomethacin, led to significant inhibition of the dilator response in both control and RV co-incubated vessel segments (* p<0.02). # RV main effect (p<0.05). Data are means ± SEM.*

Figure 2: Effects of resveratrol (RV) of the dilator response to intraluminal flow. ***A) A representative flow trace. B)*** *Incubation with RV significantly reduced the dilator response of isolated pressurized femoral arteries from 4-(YRV, young RV; n = 10) and 26-month-old mice (ORV, old RV; n = 6) to intraluminal flow (8 and 10 μL•min^{-1}) when compared to age-matched controls (YC, young control; n = 12 and OC, old control; n = 6) (p<0.05). * Control is different from RV in both young and old at p<0.05.* ***C, D) Characterization of the dilator response to***

intraluminal flow in vessels from young and old mice. *Incubation of isolated pressurized femoral arteries from (**C**) 4-month old mice; and (**D**) 26-month old mice, with L-NG-nitro-L-arginine (L-NNA; 100 μM; n = 6), indomethacin (10 μM; n = 6), or apamin (100 nM) + Tram 34 (1 μM) (Young, n = 6; Old n = 8) all reduced flow-mediated dilation in both young and old. Data are means ±SEM; Treatment symbols in figure 2C are identical to those indicated in figure 2B. * Higher at 10 μL•min^{-1} than at 5 μL•min^{-1} (p<0.05); # Control (PSS) different from Indomethacin and L-NNA (p<0.01); $ Control (PSS) different from apamin +Tram 34 (p<0.05).*

Figure 3: Effects of resveratrol (RV) on dilation in femoral arteries from eNOS knockout mice. ***A) effect of RV on acetylcholine (ACh)-induced dilation:*** *Incubation of the isolated femoral artery segments from eNOS KO mice (n=4) with RV improved dilation to levels similar to that observed in femoral arteries from wildtype mice and significantly higher than in the presence of potassium channel inhibitors apamin + Tram34.* ***B) Effect of RV on dilator response to flow****: Incubation of the artery segments from eNOS KO mice in RV (n=4) or RV plus apamin & Tram34 inhibitors (n=4) had no overall effect on FMD. Controls included incubation in PSS for eNOS KO mice (n=4) and wildtype C57 mice (n=15). * Different from RV plus inhibitors at p<0.05 and ** at p<0.01. Data are presented as mean +- SEM.*

Figure 4: Effects of resveratrol (RV) on NO_2 production by cultured human coronary artery endothelial cells (HCAEC). *(**A**) Pre-incubation with RV for one hour increased the production of NO_2 by cultured HCAEC in response to ACh (10^{-5} M) stimulation for two minutes when compared to controls (p<0.05). (**B**) With the exception of flow rates of 8 μL•min-1, pre-*

*incubation with RV (45 μM) for one hour reduced the production of NO_2 by cultured HCAEC in response to increased flow (5 & 10 μL•min^{-1}), particularly during the first three minutes of exposure ($p<0.05$). Data are means ± SEM. (**C**) Representative images of the HCAEC Live-Dead assay. Cultured cells were incubated with growth medium (controls; A) or RV for one hour (B). Following incubation, both groups of cells were exposed for 2 (C), 10 (D), and 60 minutes (E) to acetylcholine (ACh; 10^{-5} M). Different sets of cells (controls and RV) were exposed to increased flow (5-10 μL ·min^{-1}) for three (F; 5 μL ·min^{-1}), six (G; 8 μL ·min^{-1}) and nine minutes (H; 10 μL · min^{-1}). Dead cells were stained with EthD-1 (red); live cells were stained with Calcein-AM (green). No treatment (ACh, increased flow or RV) had negative effects on cell viability in relation to controls. Cell viability (%) for each group was (mean ± SD) A:99 ± 1;B: 98 ± 1;C: 95 ± 1;D: 97 ± 1;E: 94 ± 1; F: 97 ± 2;G: 94 ± 1 and H: 93 ± 1*.

Figure 1

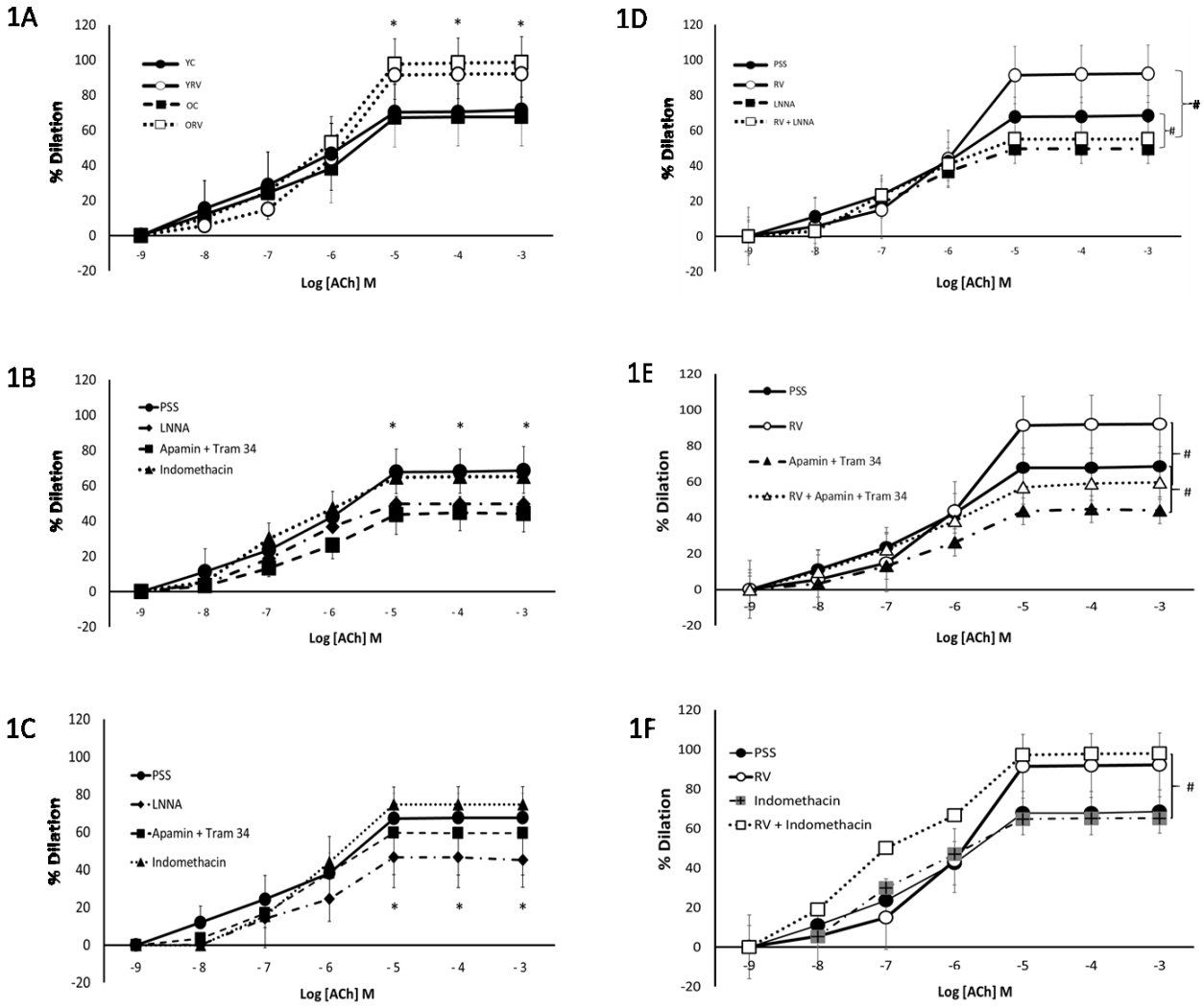
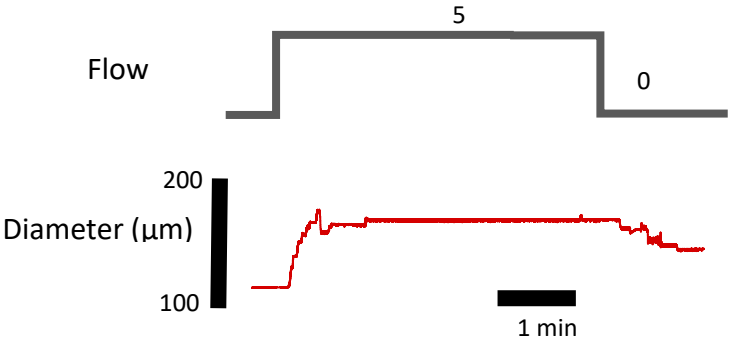

1A
% Dilation
Log [ACh] M
YC
YRV
OC
ORV
1D
PSS
RV
LNNA
RV + LNNA
1B
PSS
LNNA
Apamin + Tram 34
Indomethacin
1E
PSS
RV
Apamin + Tram 34
RV + Apamin + Tram 34
1C
PSS
LNNA
Apamin + Tram 34
Indomethacin
1F
PSS
RV
Indomethacin
RV + Indomethacin

Figure 2

A


5
Flow
0
200
Diameter (µm)
100
1 min

B

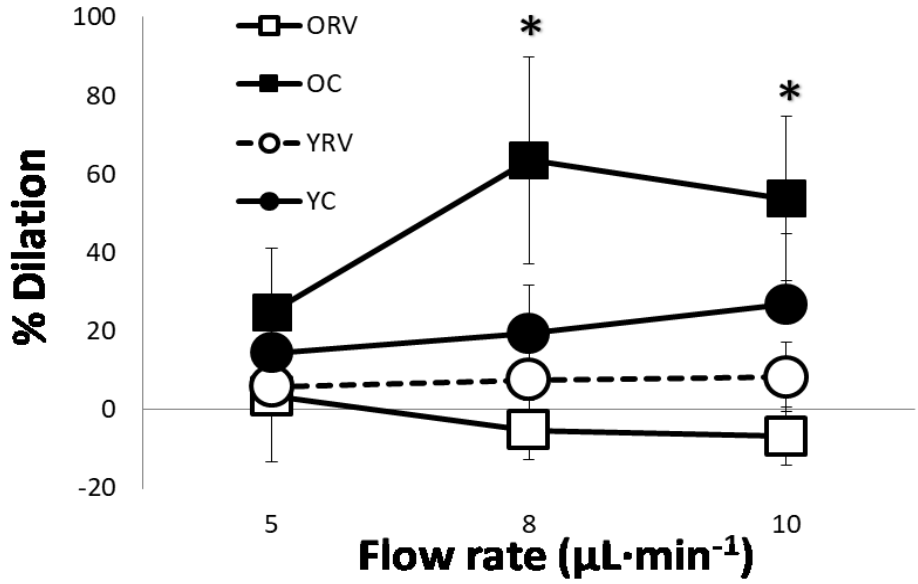
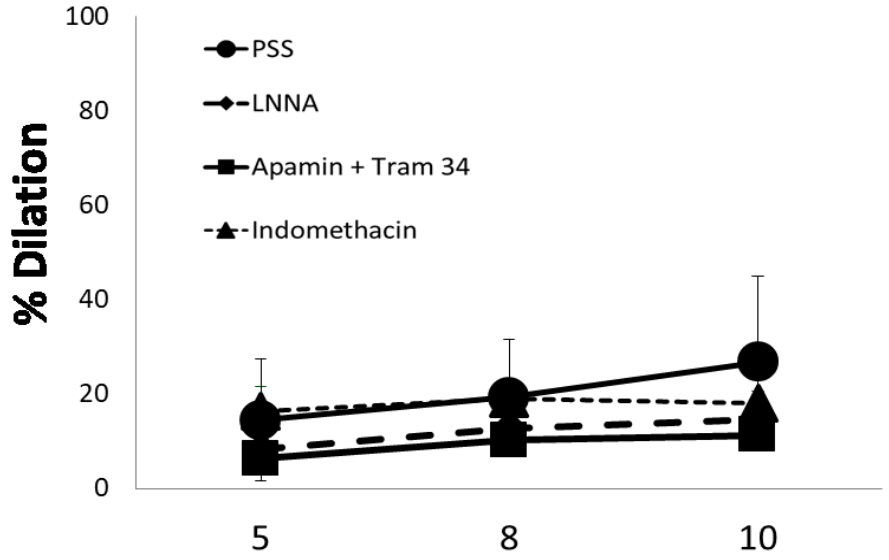

100
80
60
40
20
0
-20
% Dilation
ORV
OC
YRV
YC
*
*
5
8
10
Flow rate (µL·min-1)

Figure 2

C


100
80
60
40
20
0
% Dilation
PSS
LNNA
Apamin + Tram 34
Indomethacin
5
8
10

D

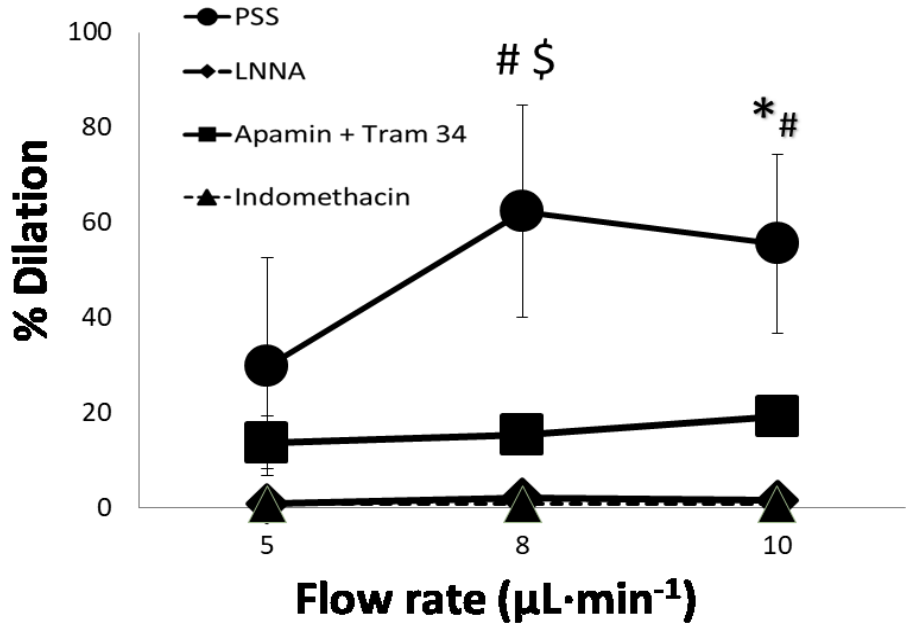
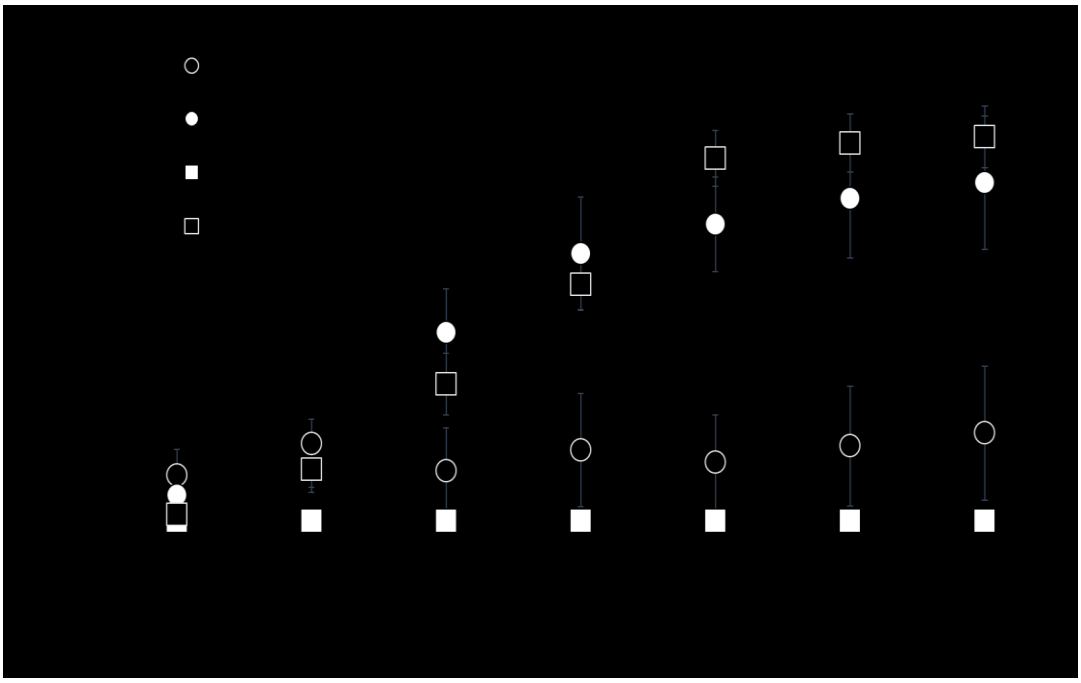

100
80
60
40
20
0
% Dilation
PSS
LNNA
Apamin + Tram 34
Indomethacin
$
*#
5
8
10
Flow rate (µL·min-1)

Figure 3

A



B

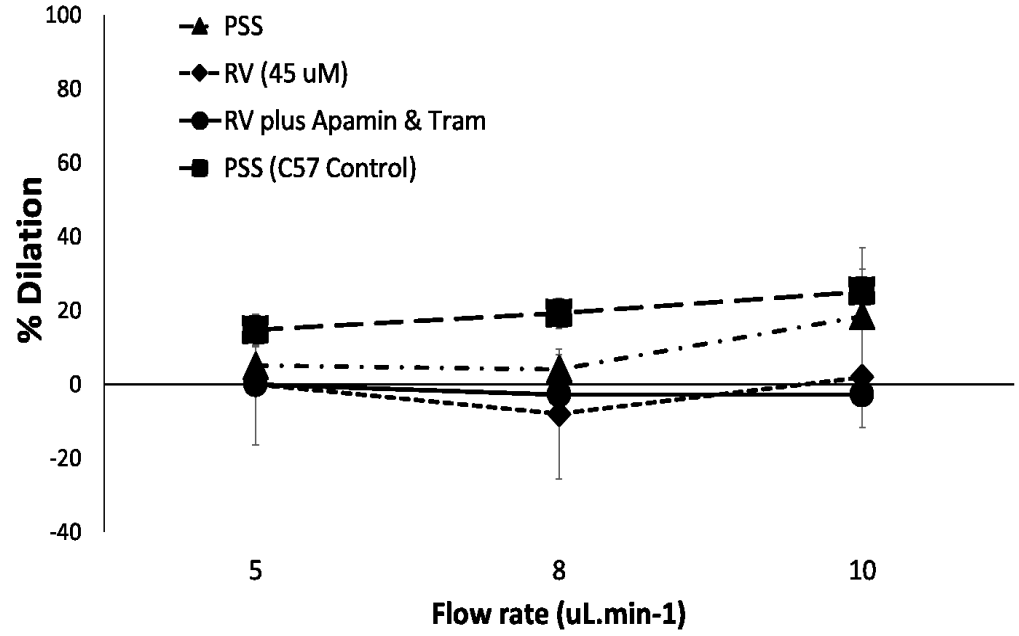
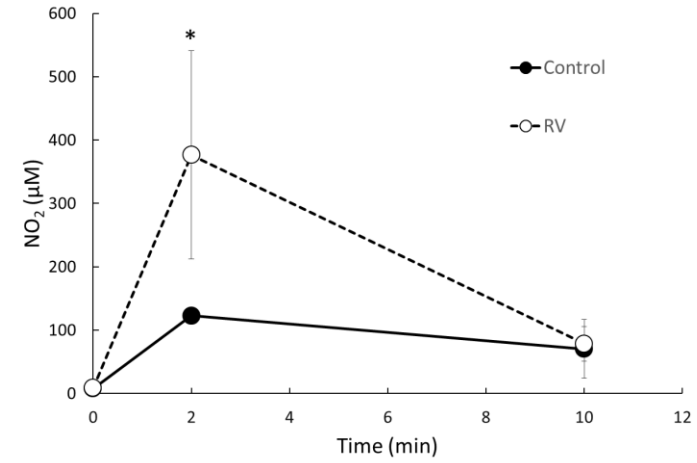
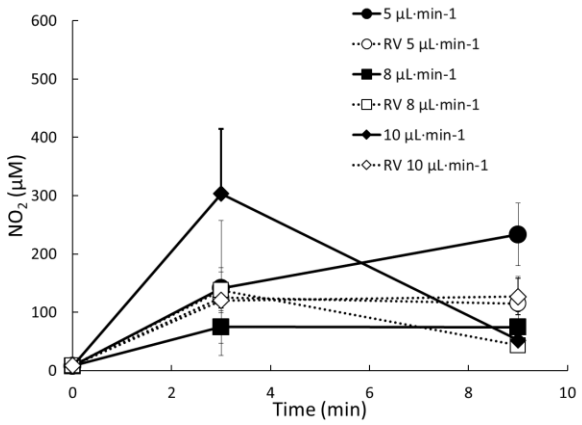

PSS
RV (45 uM)
RV plus Apamin & Tram
PSS (C57 Control)
% Dilation
100
80
60
40
20
0
-20
-40
5
8
10
Flow rate (uL.min-1)

Figure 4

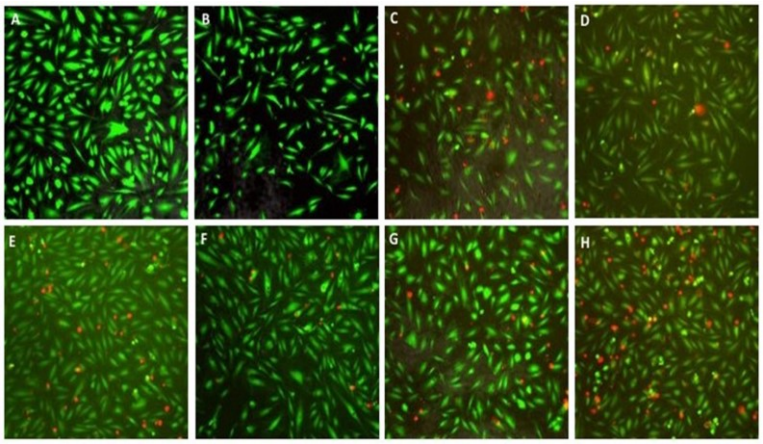
A


600
500
400
300
200
100
0
NO_2 (µM)
*
Control
RV
0
2
4
6
8
10
12
Time (min)

B


600
500
400
300
200
100
0
NO_2 (µM)
5 µL·min-1
RV 5 µL·min-1
8 µL·min-1
RV 8 µL·min-1
10 µL·min-1
RV 10 µL·min-1
0
2
4
6
8
10
Time (min)

C


A
B
C
D
E
F
G
H